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13. Fibroblast cell cultures were grown in Dulbecco modified Eagle medium (DMEM) supplemented with fetal calf serum (FCS, 10%), and 2 mM L-glutamine. Selective medium contained xanthine (250 $\mu\text{g ml}^{-1}$), hypoxanthine (15 $\mu\text{g ml}^{-1}$), and mycophenolic acid (6 $\mu\text{g ml}^{-1}$). For studies of serum-responsive gene expression, cells were seeded on 100-mm tissue culture dishes (Nunc) at approximately 5×10^5 cells per dish, grown to confluence in 10% FCS, then maintained in 1% FCS 3 to 5 days (serum-starved (-)). Starved cultures were stimulated with 15% FCS for 1 hour, unless otherwise indicated [serum-stimulated (+)]. AW-Ramos tumor cells were grown in RPMI supplemented with FCS (10%), 2 mM L-glutamine, and 50 μM 2-mercaptoethanol.
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Mutants of Pertussis Toxin Suitable for Vaccine Development

MARIAGRAZIA PIZZA, ANTONIO COVACCI, ANTONELLA BARTOLONI, MARIA PERUGINI, LUCIANO NENCIONI, MARIA TERESA DE MAGISTRIS, LUIGI VILLA, DANIELE NUCCI, ROBERTO MANETTI, MASSIMO BUGNOLI, FRANCO GIOVANNONI, ROBERTO OLIVIERI, JOSEPH T. BARBIERI, HIROKO SATO, RINO RAPPUOLI*

Immunization with chemically detoxified pertussis toxin can prevent severe whooping cough with an efficacy similar to that of the cellular pertussis vaccine, which normally gives unwanted side effects. To avoid the reversion to toxicity and the loss of immunogenicity that may follow chemical treatment of pertussis toxin, inactive toxins were constructed by genetic manipulation. A number of genetically engineered alleles of the pertussis toxin genes, constructed by replacing either one or two key amino acids within the enzymatically active S1 subunit, were introduced into the chromosome of strains of *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica*. These strains produce mutant pertussis toxin molecules that are nontoxic and immunogenic and that protect mice from the intracerebral challenge with virulent *Bordetella pertussis*. Such molecules are ideal for the development of new and safer vaccines against whooping cough.

WHOOPING COUGH, AN ACUTE respiratory disease affecting over 60 million infants and responsible for approximately 1 million deaths each year, can be prevented by vaccination (1). The vaccine currently used, containing killed *Bordetella pertussis* cells (2), although effective, has been associated with rare neurological complications and deaths (3), which have decreased the acceptance of the vaccine in many countries (4). As a result, pressure has built up to develop safer, acellular vaccines against whooping cough (4), and a number of vaccines have been proposed.

Pertussis toxin (PTX), a major virulence factor of *Bordetella pertussis*, either alone or combined with other antigens, is the main component of all acellular vaccines so far developed (5). A large-scale clinical trial carried out in Sweden has shown that chemically detoxified PTX can prevent severe whooping cough with an efficacy equal to or close to that of the cellular vaccine (6). Unfortunately, the chemical methods used to detoxify PTX are not completely satisfactory, since they give a product with reduced immunogenicity, which in some cases has been shown to revert to toxicity (7). Complete and stable detoxification of PTX is

mandatory, since the severe complications of the cellular vaccine that can lead to permanent neurological damage and death may be due to minute traces of residual toxin activity (8). The approach that we have adopted is aimed at genetic detoxification.

Pertussis toxin is a complex bacterial protein toxin composed of five noncovalently linked subunits called, according to their

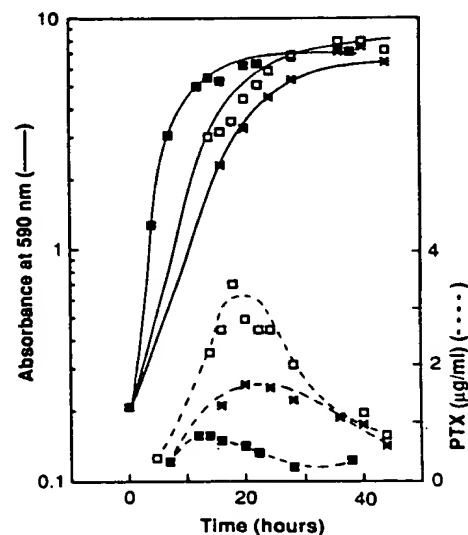
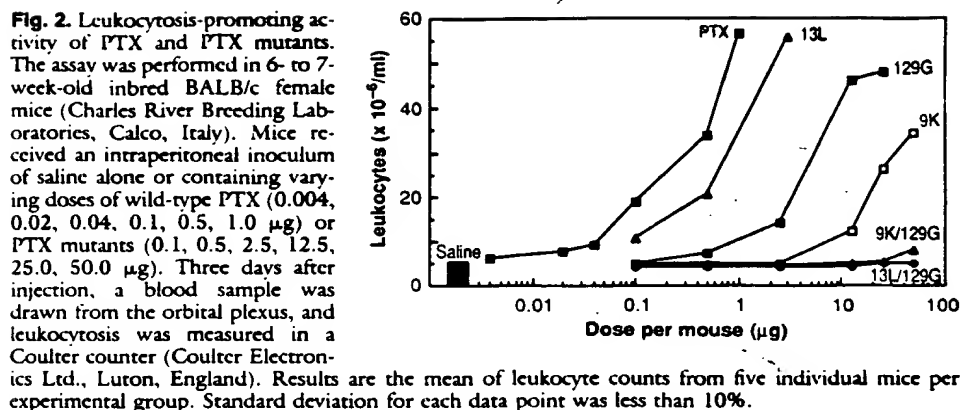


Fig. 1. Production of PTX-129G by *B. pertussis* W28 (X), *B. parapertussis* P14 (□), and *B. bronchiseptica* 7865 (■). Bacteria were grown in 125-ml flasks containing 15 ml of Steiner-Scholte medium at 35°C for 72 hours. The amount of PTX present in the supernatant was determined by enzyme-linked immunoassay (11). Standard deviation for each data point was less than 15%. The other PTX mutants listed in Table 1 were also produced by the three *Bordetella* species with a similar pattern.

M. Pizza, A. Covacci, A. Bartoloni, M. Perugini, L. Nencioni, M. T. De Magistris, L. Villa, D. Nucci, R. Manetti, M. Bugnoli, F. Giovannoni, R. Olivieri, R. Rappuoli, Sclavo Research Center, Via Fiorentina 1, 53100 Siena, Italy.
J. T. Barbieri, Department of Microbiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226.
H. Sato, National Institute of Health, 10-35, 2-Chome, Kamiosaki, Shinagawa-ku, Tokyo, Japan.

*To whom correspondence should be addressed.



electrophoretic mobility, S1 (26.220 kD), S2 (21.920 kD), S3 (21.860 kD), S4 (12.060 kD), and S5 (11.770 kD) (9). As in the case of other bacterial protein toxins, including diphtheria toxin, cholera toxin, and *Pseudomonas* exotoxin A, the toxicity of PTX is mediated by an enzymatically active subunit (S1) with adenosine diphosphate (ADP)-ribosyltransferase activity (10). When S1 binds nicotinamide adenine dinucleotide (NAD) and transfers the ADP-ribose moiety to a family of eukaryotic guanosine triphosphate (GTP) binding proteins (G proteins) involved in transmembrane signaling, the response of eukaryotic

cells to exogenous stimuli is altered (10). The remaining subunits, S2, S3, S4, and S5, present in a 1:1:2:1 ratio, form a nontoxic oligomer that binds the receptors on the surface of eukaryotic cells and allows the toxic subunit S1 to reach its intracellular target proteins.

We cloned the genes coding for the five subunits of PTX from the chromosome of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* and determined their nucleotide sequences (11). The five genes are clustered in an operon in the following order: S1, S2, S4, S5, and S3. Expression of the PTX genes requires a promoter region of 170 bp and

trans-acting factors encoded by a locus called *vir*. *Bordetella parapertussis* and *B. bronchiseptica* have a defective promoter region and do not produce PTX (12).

Development of recombinant vaccines turned out to be more difficult than anticipated, because *Escherichia coli* is unable to express and assemble pertussis holotoxin (13). High-yield expression of the individual subunits was eventually achieved in *E. coli* and *Bacillus subtilis* and the recombinant S1 subunit retained its enzymatic activity (13, 14). However, none of the recombinant subunits alone were able to induce protective immunity, possibly because their conformation is different from that found in the holotoxin. Therefore, we and others have used the *E. coli* system to identify the amino acids of the S1 subunit that are essential for the enzymatic activity, with the aim of substituting their codons in the chromosome of *B. pertussis* (15).

We describe here the construction of *Bordetella* strains that synthesize and release into the culture medium forms of pertussis toxin with no detectable toxicity. These molecules are immunogenic and induce protective immunity in animal models.

Substitution of either Arg⁹ with Lys, Arg¹³ with Leu, or Glu¹²⁹ with Gly abolishes the enzymatic activity of the recombinant S1 subunit without impairing its ability to be recognized by protective monoclonal antibodies (15). By using homologous recombination (16), we replaced the chromosomal PTX operon of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* with an operon containing a functional promoter and coding for a PTX protein in which the amino acid substitutions described above were introduced by in vitro mutagenesis. All three species can express and release into the culture medium the mutated forms of PTX (Fig. 1). This demonstrates that *B. parapertussis* and *B. bronchiseptica*, which normally do not produce PTX, can produce it when provided with an active promoter. Under these conditions, *B. parapertussis* is a better producer of PTX and, since it grows faster than *B. pertussis*, should be suitable for large-scale production of mutant molecules.

The mutant *B. pertussis* strains obtained by recombination of the PTX operon were grown in 5- or 20-liter fermenters, and the mutant PTX molecules were recovered from the culture supernatant by Affi-gel blue absorption and were purified on a tetrahydrofuran-Sepharose affinity column (11). The purified PTX molecules separated by electrophoresis on SDS-acrylamide gel were indistinguishable from the wild-type PTX (17). In contrast, the ADP-ribosyltransferase activity was reduced by a factor of 1000, 200, and 50 in the three PTX mutants (Table 1).

Table 1. Properties of PTX and the mutant PTX molecules: PTX-9K, Arg⁹→Lys; PTX-13L, Arg¹³→Leu; PTX-129G, Glu¹²⁹→Gly; PTX-9K/129G, Arg⁹→Lys and Glu¹²⁹→Gly; PTX-13L/129G, Arg¹³→Leu and Glu¹²⁹→Gly. The data shown are the result of at least three determinations and the standard deviations for each set of data were usually below 10% and never more than 15%. Clustering activity on CHO cells was determined as described (18). The minimum clustering dose of PTX was 5 μ g/ml. The double mutants were tested up to a concentration of 5 μ g/ml without detection of any clustering activity. The ADP-ribosylation of transducin assay was performed as described (15) with scalar amounts of PTX (from 5 to 500 ng) or PTX mutants (1 to 20 μ g). The minimum amount of PTX detected by the assay was 1 to 5 ng. The double mutants did not show any activity up to 20 μ g, the maximum amount of protein that could be used in this test. The affinity constant was determined by competitive radioimmunoassay in microtiter plates coated either with the monoclonal antibody 1B7 (19) or goat γ -globulins against PTX. Each sample was analyzed in duplicate. The data were analyzed by a nonlinear regression analysis (21) and the affinity constant calculated as described (21). ND, not done. Human T cell clones, recognizing different epitopes on the wild-type S1 subunit (20), were incubated with mitomycin C-treated autologous antigen-presenting cells and heat-denatured toxoids at 3 μ g/ml. The proliferation assays were performed as described (20). The results obtained with two clones, T215 and S223, recognizing amino-terminal and carboxyl-terminal epitopes, respectively, are shown in the table. Background proliferations were 0.9 and 0.6 counts per minute (cpm) $\times 10^3$ for T215 and S223, respectively. The PTX double mutants were also tested with clones S106, S105, T216, and T219 (20) with similar results.

PTX mutant	Production (μ g/ml)	Toxicity CHO cells (%)	ADP-ribosylation (%)	Antibody recognition (affinity constant)		T cell recognition (10^3 cpm)	
				Protective γ -globulins ($\times 10^{-10}$)	Monoclonal 1B7 ($\times 10^{-8}$)	T215	S223
PTX	4.1	100	100	5.0	3.5	8.2	6.6
PTX-9K	3.2	0.1	0.10	1.0	8.9	14.2	9.2
PTX-13L	3.7	25	2.00	ND	ND	14.9	7.3
PTX-129G	3.1	10	0.50	1.7	2.1	18.6	18.8
PTX-9K/129G	2.7	<0.0001	<0.01	1.2	3.3	17.0	8.8
PTX-13L/129G	2.0	<0.0001	<0.01	1.3	1.3	19.6	10.0

Table 2. Vaccine potency: mouse survival in response to the intracerebral challenge. Mice were immunized via intraperitoneal route either with the suitable dilution of the cellular vaccine [the standard cellular vaccine was provided by the National Institutes of Health (Bethesda) and contained eight international protective units per milliliter] or they were immunized intraperitoneally with scalar doses of the PTX double mutants diluted in saline. After 15 days they were challenged intracerebrally with the *B. pertussis* strain 18323. The table reports the survival of mice 2 weeks after challenge.

No.	standard cellular vaccine	Acellular vaccines		
		Dose (μ g)	Survivors	
	Survivors		9K/129G	13L/129G
0	15/16	30	16/16	16/16
0	13/16	12	16/16	16/16
0	9/16	4.8	12/16	16/16
0	2/16	1.92	10/16	15/16
0		0.77	7/16	11/16
0		0.25	3/16	4/16

They were tested for their ability to cluster Chinese hamster ovary (CHO) cells (18), all mutants showed a reduced but significant residual toxicity which ranged from 0.1% to 2% of the wild-type PTX (Table 1). The toxicity was even more evident *in vivo* in the mouse leukocytosis assay (18), where even the least toxic molecule (PTX-9K), at the dose of 50 μ g per mouse, could induce a significant increase in leukocyte number (Table 2). These results indicate that, whereas the mutations introduced resulted in reduced enzymatic activity and toxicity of the PTX molecules, the toxicity of all mutants was still too high to be suitable for vaccine production. We therefore introduced two of the described mutations together in a single S1 subunit and obtained *Bordetella* strains expressing S1 double mutants (PTX-9K/129G or PTX-13L/129G). Also these new proteins, when separated by electrophoresis on SDS-acrylamide gels, are indistinguishable from the wild-type toxin (17). However, the treatment of CHO cells with up to 5 μ g of the double-mutant proteins per milliliter did not show detectable toxicity. Furthermore, none of the mutant proteins were toxic in the mouse leukocytosis assay up to a dose of 50 μ g per mouse, which is a dose 100 times higher than the median lethal dose of PTX (Fig. 2). The mutant proteins did not show potentiation of anaphylaxis nor stimulation of histamine sensitivity in mice (17), which are activities typical of PTX (18). ADP-ribosyltransferase activity could not be detected *in vitro* (Table 1). In addition to the mutants described above, we generated more than 30 strains of *Bordetella* in which most of the mutations known to reduce the enzymatic activity of the S1 subunit (15) were introduced, alone or in combination, and resulted in double or triple mutants. However, most of them were not useful because the mutant PTX proteins were either produced in low yield, not assembled, or found to be toxic. In conclusion, only the mutant proteins we described

were essentially nontoxic and produced in reasonable amounts so that they represent suitable candidates for vaccine development, provided that their antigenic and immunogenic properties are similar to those of PTX.

To test the antigenic properties of the PTX double mutants, we used them to compete with the binding of 125 I-labeled PTX to a monoclonal antibody, previously described, that recognizes a conformational protective epitope of the S1 subunit (19). The same mutant proteins were also used to compete with the binding of 125 I-labeled PTX to a polyclonal antiserum against PTX that neutralizes the toxin *in vitro* and recognizes all five PTX subunits in an immunoblot. All mutant proteins showed the same affinity as wild-type PTX in the competition experiment against polyclonal and monoclonal antibodies to PTX (Table 1). The mutant PTXs were also challenged for their recognition by human T cell clones previously shown to be specific for immunodominant T cell epitopes of the S1 subunit (20). As shown in Table 1, the T cell clones gave a proliferative response in the presence of the mutant PTX and autologous antigen-presenting cells. This result indicates that the immunodominant T cell epitopes of the S1 subunit from both the wild-type and mutant PTX are identical and can be processed by the antigen-presenting cells with the same efficiency. We conclude that the antigenic structure of the holotoxin is the same in both wild-type and mutant proteins and that the immunodominant B and T cell epitopes of the S1 subunit are not altered in the double-mutant PTX.

The immunogenicity of the PTX mutants was tested by immunizing rabbits with the mutant protein according to the protocol described by Nicosia *et al.* (13). The antisera obtained can neutralize the CHO cell clustering activity of 5 ng of PTX per milliliter up to a dilution of 1 to 100, the same titer previously obtained with the wild-type PTX (17). Finally, we tested the ability of the

PTX double mutants to protect mice from the intracerebral challenge with virulent *B. pertussis*, an assay used to test the efficacy of the cellular pertussis vaccine (18). Table 2 shows that the two mutants protect mice in a dose-dependent fashion, with 100% survival, down to 4.8 to 12 μ g of mutant protein per mouse.

In conclusion, the PTX mutants described here represent ideal candidates for the development of a safer vaccine against whooping cough.

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Human Cells Lacking mtDNA: Repopulation with Exogenous Mitochondria by Complementation

MICHAEL P. KING AND GIUSEPPE ATTARDI

Two human cell lines (termed ρ^0), which had been completely depleted of mitochondrial DNA (mtDNA) by long-term exposure to ethidium bromide, were found to be dependent on uridine and pyruvate for growth because of the absence of a functional respiratory chain. Loss of either of these two metabolic requirements was used as a selectable marker for the repopulation of ρ^0 cells with exogenous mitochondria by complementation. Transformants obtained with various mitochondrial donors exhibited a respiratory phenotype that was in most cases distinct from that of the ρ^0 parent or the donor, indicating that the genotypes of the mitochondrial and nuclear genomes as well as their specific interactions play a role in the respiratory competence of a cell.

MITOCHONDRIAL BIOGENESIS IS under the control of both the nuclear and the mitochondrial genome (1). The replication and transcription of mtDNA and the translation of mtDNA-coded mRNAs require components encoded in the nucleus, which must be imported into the organelles. Most of the enzymes of the respiratory chain are oligomeric complexes consisting of both nuclear DNA-coded and mtDNA-coded subunits. Study of these nuclear-mitochondrial interactions would be facilitated if it were possible to manipulate the mtDNA complement of a cell, move mitochondria from one cellular environment to another, or introduce new genes into mitochondria.

We have isolated two derivatives of the human cell line 143B.TK⁻, which had been entirely depleted of mtDNA by long-term exposure to low concentrations of ethidium bromide (2). DNA transfer hybridization analysis of total DNA from these cell lines, designated 143B101 and 143B206 (or ρ^0 101 and ρ^0 206), did not reveal the expected mtDNA restriction fragments, under conditions in which much less than one molecule per cell would have been detected (2). These ρ^0 cells rely exclusively on glycolysis for their energy requirements, and, as previously shown for ρ^0 avian cells (3), have become pyrimidine auxotrophs because of the deficiency of the respiratory chain-dependent dihydroorotate dehydrogenase (4). Unexpectedly, these ρ^0 cells have also be-

come pyruvate-dependent (2). Complementation of either of these metabolic requirements was used as a selectable marker for the repopulation of ρ^0 cells with exogenous functional mtDNA.

The ρ^0 cell line 143B206 undergoes less than one population doubling in the absence of uridine, whereas its growth rate

closely approaches that of the parental line in the presence of uridine at 50 μ g/ml (Fig. 1A). The growth rate of the 143B parental line is not affected by the absence of uridine. When 5×10^6 143B206 cells were grown in the absence of uridine, no colony appeared even after 10 weeks of selection in culture. These ρ^0 cells have a similar dependence on pyruvate [a normal component of the Dulbecco modified Eagle's medium (DMEM)] for growth, undergoing less than one population doubling in the absence of pyruvate (Fig. 1B). In the presence of pyruvate at 100 μ g/ml, the growth rate of this cell line is similar to that of the parental line, whose growth is not affected by the absence of pyruvate. An identical dependence on uridine and pyruvate was observed with the 143B101 cell line (2).

We used the two ρ^0 cell lines for mitochondrial transformation studies. Initially, mitochondria were transferred by fusion of cytoplasts with ρ^0 cells. Cybrids were formed by fusion of cytoplasts from HT1080-6TG human cells (enHT1080) (5) with ρ^0 101 or ρ^0 206 cells and replated in medium containing bromodeoxyuridine (BrdU) and lacking either pyruvate or uridine. These media permitted only the growth of ρ^0 cells that had fused with cytoplasts containing functional mitochondria. Growth of presumptive transformants was observed within 3 to 4 days after cell fusion, and colonies were picked (with a

Fig. 1. Growth of (A and B) 143B.TK⁻ (Par) cells or 143B206 (ρ^0 206) cells and (C) transformants 206.417.U3 and (D) transformant 206.415.P3 in the presence or absence of uridine (U) or pyruvate (P). (A and B) Multiple series of 10-cm plastic petri dishes were seeded each with a constant number of cells in (A) 10 ml of normal DMEM supplemented with 5% dialyzed fetal bovine serum (FBS), BrdU at 100 μ g/ml, and uridine (U) at 0 or 50 μ g/ml, or in (B) 10 ml of pyruvate-deficient DMEM supplemented with 5% dialyzed FBS, BrdU at 100 μ g/ml, uridine at 50 μ g/ml, and pyruvate (P) at 0 or 100 μ g/ml. At various time intervals, cells from individual plates were trypsinized and counted. (C and D) Growth curves of the recipient ρ^0 206 cells and of the transmitochondrial cell lines were determined as described above. The growth medium was normal DMEM supplemented with 5% dialyzed FBS and BrdU at 100 μ g/ml (+P, -U), or pyruvate-deficient DMEM supplemented with 5% dialyzed FBS and BrdU at 100 μ g/ml (-U, -P), or the latter medium plus uridine at 50 μ g/ml (+U, -P), or the same medium plus uridine at 50 μ g/ml and pyruvate at 110 μ g/ml (+U, +P). The transformants had been maintained in their original selective media for 5 weeks (206.415) and 7 weeks (206.417) after fusion. The transmitochondrial derivatives were obtained by fusing 143B206 cells with cytoplasts from HT1080-6TG. For preparation of cytoplasts, HT1080-6TG cells were plated on 35-mm dishes at $\sim 2 \times 10^5$ cells per dish. After ~ 24 hours, cells were enucleated while attached to the plate (15). The cytoplasts were fused as a monolayer with 3×10^5 to 8×10^5 143B206 cells (16), and incubated in DMEM supplemented with 5% FBS and uridine at 50 μ g/ml. The cells were replated 1 to 3 days after fusion and placed in selective medium. Selective uridine-free medium consisted of DMEM supplemented with 5% dialyzed FBS and BrdU at 100 μ g/ml, and selective pyruvate-free medium consisted of DMEM lacking pyruvate supplemented with 5% dialyzed FBS, uridine at 50 μ g/ml, and BrdU at 100 μ g/ml.

